The Plant Journal (2002) **32**, 905–914

Tomato *tos1* mutation identifies a gene essential for osmotic tolerance and abscisic acid sensitivity

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Summary

Osmotic stress severely limits plant growth and agricultural productivity. We have used mutagenesis to identify plant genes that are required for osmotic stress tolerance in tomato. As a result, we have isolated a novel mutant in tomato (*tos1*) caused by a single recessive nuclear mutation that is hypersensitive to general osmotic stress. Growth measurements demonstrated that the *tos1* mutant is less sensitive to intracellular abscisic acid (ABA) and this decreased ABA sensitivity of *tos1* is a basic cellular trait expressed by the mutant at all developmental stages analysed. It is not caused by a deficiency in the synthesis of ABA because the *tos1* seedlings accumulated more ABA than the wild type (WT) after osmotic stress. In contrast, the *tss2* tomato mutant, which is also hypersensitive to osmotic stress, is hypersensitive to exogenous ABA. Comparative analysis of *tos1* and *tss2* indicates that appropriate ABA perception and signalling is essential for osmotic tolerance.

Keywords: abscisic acid, osmotic stress, *tos1* mutant, *tss2* mutant.

Introduction

Osmotic stress caused by drought is one of the most detrimental environmental stresses limiting plant productivity (Bohnert *et al.*, 1995; Boyer, 1982). Osmotic stress can also be caused by high salt (the most common of which is NaCl) concentration in the soil that produces a decrease in water potential which, in turn, affects water availability to the plant (Hasegawa *et al.*, 2000). In addition to the hyperosmotic shock, and the subsequent oxidative stress generated (Borsani *et al.*, 2001b), the deleterious consequences of high NaCl concentration in the external solution of plant cells also includes ion toxicity and nutrient imbalance (Hasegawa *et al.*, 2000; Niu *et al.*, 1995; Rodriguez-Navarro, 2000; Serrano *et al.*, 1999). Changes in plant metabolism and hormone levels occur in response to the osmotic stress and the ionic toxicity caused by salt stress (Bohnert *et al.*, 1995; Bray, 1997; Cuartero and Fernández-Muñoz, 1999). However, the contribution of each of these two NaCl stress components to stress symptoms, as well as their interaction, remains unclear.

Because salt stress can be applied accurately and reproducibly, osmotic stress induced by NaCl instead of the osmotic stress caused by drought has been commonly used in the laboratory to study the plant response to osmotic stress (Zhu, 2000). Thus, stress induced by NaCl has been employed in yeast in order to identify many components involved in the signal transduction pathway leading to osmotolerance (Toone and Jones, 1998). Recently, a number of NaCl-hypersensitive mutants have been isolated in *Arabidopsis* and tomato (Borsani *et al.*, 2001a; Zhu *et al.*, 1998). These mutants defined genes and processes that are essential for salt tolerance. As a result, four *sos* (for overly salt-sensitive) mutants in *Arabidopsis* and two *tss* (for tomato salt-sensitive) mutants in tomato have been identified (Borsani *et al.*, 2001a; Liu and Zhu, 1997; Shi *et al.*, 2002; Wu *et al.*, 1996; Zhu *et al.*, 1998). Mutations in these genes render the *sos* and *tss* plants hypersensitive to the ionic component of NaCl stress with no significant effect on osmotic tolerance. The exception is the *tss2* mutant, which is hypersensitive to both ionic and osmotic stresses. Characterisation of *tss2* suggested that signalling by the plant hormone, abscisic acid (ABA) is important for salt and/or osmotic plant tolerance, because *tss2* is hypersensitive to growth inhibition by ABA (Borsani *et al.*, 2001a).

ABA plays a major role in plant adaptation to high salinity and osmotic stress (Leung and Giraudat, 1998; Zhu, 2002).

During vegetative growth, endogenous ABA levels increase upon conditions of water stress, and the increased ABA is an essential mediator in triggering the plant response (Bray, 1997; Chandler and Robertson, 1994; Leung and Giraudat, 1998; Skriver and Mundy, 1990; Zhu, 2002). Genetic screens have led to the isolation of several plant mutants with altered ABA responsiveness (Leung and Giraudat, 1998; Møller and Chua, 1999). They can be classified into three classes: (i) biosynthetic mutants that possess severely reduced amounts of the hormone (*aba*); (ii) and mutants that either exhibit insensitivity (*abi*); (iii) or hypersensitivity (*era*, *sad1* and *abh1*) to ABA. In *Arabidopsis* in particular, mutations in all five *ABI* loci reduce the sensitivity of seed germination to exogenous ABA (Finkelstein, 1994; Koornneef *et al*., 1984; Leung and Giraudat, 1998). In contrast, the *Arabidopsis era*, *sad1* and *abh1* mutants display hypersensitivity to ABA during germination (Cutler *et al*., 1996; Hugouvieux *et al*., 2001; Xiong *et al*., 2001).

The proteins encoded by ABI3, ABI4 and ABI5 have characteristics of transcriptional regulators (Finkelstein and Lynch, 2000; Finkelstein *et al*., 1998; Giraudat *et al*., 1992; Lopez-Molina and Chua, 2000). *ABI1* and its homologue, *ABI2*, encode type 2C serine/threonine phosphatases (Leung *et al*., 1994; Meyer *et al*., 1994; Rodriguez *et al*., 1998). The identification of intragenic revertants in the *ABI1* gene demonstrated that a loss of ABI1 phosphatase activity leads to an enhanced responsiveness to ABA indicating that the wild-type (WT) ABI1 and ABI2 proteins are negative regulators of ABA responses (Gosti *et al*., 1999). *ERA1* encodes a farnesyl transferase that may play a role in embryonic ABA signalling (Cutler *et al*., 1996). *ERA3* is allelic to the *ETHYLENE INSENSITIVE2* (*EIN2*) gene demonstrating a clear interaction between ABA and ethylene signalling pathways (Ghassemian *et al*., 2000). *ABH1* encodes for a nuclear mRNA cap-binding protein (Hugouvieux *et al*., 2001) and *SAD1* encodes for a protein with similarity to Sm proteins contained in snRNPs that function in splicing (Xiong *et al*., 2001). These results involve RNA metabolism in the modulation of ABA response in *Arabidopsis* (McCourt, 2002).

All plant mutants isolated to date using NaCl are specifically hypersensitive to ionic stress. In order to identify specific osmotic hypersensitive mutants, a previously used modified mutant screening (Borsani *et al*., 2001a) in which NaCl was replaced by mannitol is used. As a result, we have isolated a tomato mutant (*tos1*), whose growth is specifically hypersensitive to osmotic stress. Interestingly, *tos1* links osmotic tolerance to ABA signalling since *tos1* exhibits decreased ABA sensitivity compared to wild-type seedlings. We have analysed *tos1* together with the previously identified *tss2* tomato mutant, hypersensitive to NaCl, osmotic stress and ABA (Borsani *et al*., 2001a). Our results indicate that both increased and decreased sensitivity to ABA lead to decreased tolerance to osmotic stress. Therefore, an appropriate ABA perception and/or signalling are required for osmotic tolerance.

Results

Isolation of a tomato mutant hypersensitive to osmotic stress

Previous screens to search plant mutants hypersensitive to general osmotic stress have failed when NaCl is used as osmotic agent. We had previously isolated three tomato mutants defining two genetic loci required for NaCl tolerance (Borsani *et al*., 2001a). Further genetic screens using NaCl resulted in the isolation of additional mutants, all of which were hypersensitive to ionic stress, but had no apparent phenotypic differences with the wild type under other osmotic stress agents (O. Borsani, V. Valpuesta and M.A. Botella, unpublished results). It is, therefore, clear that screens based on the addition of NaCl to the growing medium are biased towards the isolation of ionic, hypersensitive mutants. Our results agree with previous studies in *Arabidopsis* where all NaCl-hypersensitive mutants reported are ionic mutants involved in K^+ nutrition (Zhu *et al*., 1998; Zhu, 2000).

To design effective osmotic screens, we first studied the effect of mannitol on tomato root growth. We found that tomato seedlings were more sensitive to mannitol than to iso-osmotic concentrations of NaCl (Figure 1a). Root elongation rate was decreased by 50% at approximately 160 mM NaCl (Figure 1b) which corresponds to an osmotic potential equivalent to 333 mM mannitol (see Methods). Nevertheless, the same degree of root growth inhibition was obtained using a lower concentration of mannitol (approximately 275 mM). Similar results were obtained when fresh weight was analysed (Figure 1c). A 50% reduction in fresh weight was observed at a concentration of approximately 100 mM NaCl in the medium, corresponding to an iso-osmotic concentration of mannitol of approximately 230 mM. However, seedlings grown in 230 mM mannitol showed a reduction of 75% in fresh weight (Figure 1c). These results suggested that NaCl was not an adequate stress agent for the identification of osmotic mutants in our experimental system. Therefore, we decided to use mannitol as the osmotic stress agent in the search for osmotic hypersensitive mutants.

We screened M2 seedlings from 1300 M1 ethylmethane sulfonate-mutagenised tomato plants for root growth hypersensitivity caused by 150 mM mannitol. The same mutagenised families had been previously screened using 125 mM NaCl and resulted in the isolation of the *tss1* and *tss2* mutants (Borsani *et al*., 2001a). M2 individuals from a family with reduced tolerance to 150 mM mannitol were identified 2 days after the commencement of the treatment.

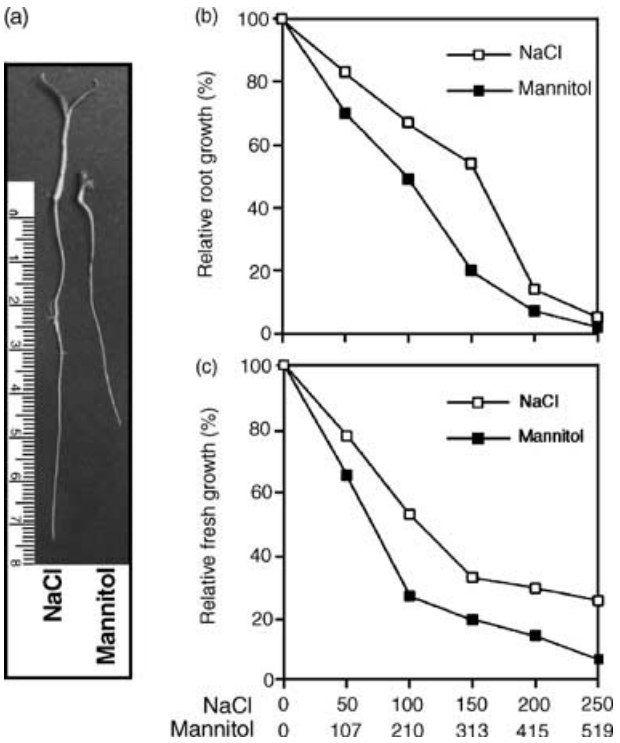

Figure 1. Tomato seedlings are more sensitive to mannitol than to iso-osmotic concentrations of NaCl.
(a) Three-day-old wild-type tomato seedlings with 1.5-cm-long roots grown on vertical agar plates on MS medium, were transferred to MS plates supplemented with 100 mM NaCl or 210 mM mannitol and photographed after 3 days (ruler in cm).
(b,c) Root elongation of wild-type tomato seedlings was measured to quantify their sensitivities to NaCl and mannitol inhibition. Seedlings were treated as above, but transferred to MS medium supplemented with the indicated concentrations of mannitol or NaCl (corresponding to iso-osmotic concentrations) and their growth was measured 3 days later. Root growth was expressed as a percentage relative to seedlings grown on MS medium. Fresh weight of wild-type tomato seedlings was measured to quantify their sensitivities to NaCl and mannitol inhibition as in (b). Fresh weight of NaCl and mannitol-treated seedlings was expressed as a percentage relative to controls incubated on MS medium.

This putative osmotic hypersensitive mutant was named tomato osmotic sensitive-1 (*tos1*). Segregation analysis of the M2 seedlings showed an approximately 3 : 1 segregation ratio of wild type to mutant. Of the 71 seedlings analysed, 54 showed a wild-type phenotype and 17 the mutant phenotype ($\chi^2 = 0.031$), indicating that the *tos1* mutation was caused by a single recessive nuclear mutation. M3 seedlings obtained from self-pollinated M2 plants which were hypersensitive to 150 mM mannitol.

TOS1 *is hypersensitive to osmotic stress and high NaCl*

We evaluated the role of *TOS1* in general osmotic stress by measuring the root elongation of seedlings placed on agar plates containing different concentrations of various osmotic agents. We have previously shown that the *tss2* mutant was hypersensitive to osmotic stress caused by mannitol, sorbitol and choline chloride (Borsani *et al.*, 2001a). On control medium, *tss2* growth was similar to that of the wild type, while *tos1* growth was already slightly reduced (Figure 2a). *tos1* and *tss2* are hypersensitive to osmotic stress caused by different concentrations of mannitol (Figure 2a). This hypersensitivity to general osmotic stress of *tos1* and *tss2* was evident when other osmotic stress agents such as sorbitol, choline chloride and proline were employed (Figure 2b,c, not shown). Taken together, these results indicate that *tos1* and *tss2* are defective in their general osmotic stress response.

To determine the sensitivity of *tos1* to NaCl, growth curves of wild type *tos1* and *tss2* were generated using NaCl (Figure 3). While both *tos1* and *tss2* were hypersensitive to NaCl concentrations over 150 mM, *tos1* was not hypersensitive to concentrations of NaCl below 100 mM NaCl. This finding explains the no identification of *tos1* in our previous screening using 125 mM NaCl (Borsani *et al.*, 2001a). This result together with the hypersensitivity of *tos1* to osmotic stress suggest that the stress caused by low concentrations of NaCl is mainly ionic, and only high concentrations of NaCl exert sufficient osmotic stress to impair root growth.

tos1 *Seedlings over-accumulate proline in response to osmotic stress*

Proline accumulation is thought to function as an osmoregulatory solute that mediates the adaptation of plants to NaCl and osmotic stresses (Delaney and Verma, 1993). Therefore, we determined whether *tos1* was affected in proline accumulation. Free proline was extracted and measured in wild-type *tos1* and *tss2* seedlings after being exposed for 3 days to either 100 mM NaCl or 210 mM mannitol, concentrations with equivalent osmotic potentials (see Methods). At 100 mM NaCl, only *tss2* was hypersensitive whereas at 210 mM mannitol, both *tos1* and *tss2* were hypersensitive. Figure 4 shows that there were no differences in proline levels among wild-type *tos1* and *tss2* grown on control media. Treatment of seedlings with 100 mM NaCl increased the levels of proline in wild-type *tos1* and *tss2* with no significant differences among them. However, in seedlings grown in 210 mM mannitol, the levels of proline in *tos1* were more than twice that of wild type (Figure 4). *tss2* also accumulated more proline than the wild type but to a lower level than *tos1* (Figure 4). The higher proline content of the seedlings after mannitol stress rather than after iso-osmotic concentrations of NaCl likely reflects the higher osmotic stress produced by mannitol. In addition, we also conclude that the osmotic hypersensitivity observed in *tos1* and *tss2* is not due to a deficiency in the accumulation of proline after osmotic stress.

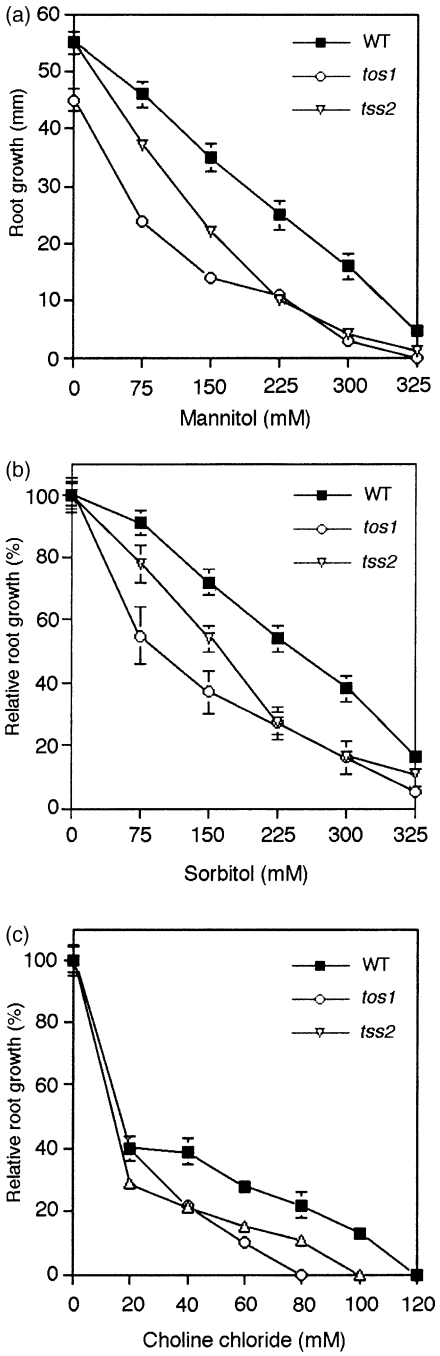

Figure 2. Sensitivity of *tos1* to different osmotic stress agents.
Root elongation of wild-type (WT) *tos1* and *tss2* seedlings was measured to quantify their sensitivities to mannitol, sorbitol and choline chloride. Seeds of wild-type *tos1* and *tss2* were germinated and grown for 3 days on MS medium. Resulting seedlings were incubated vertically on MS medium supplemented with the indicated concentrations of the stress agent, and their growth was measured 2 days later. Root growth of sorbitol and choline chloride-treated seedlings was expressed as a percentage relative to wild-type controls incubated on MS medium. Error bars represent SD ($n = 10$). The experiment was repeated at least thrice with similar results. The measurements from one representative experiment are presented.
(a) Growth response to mannitol.
(b) Growth response to sorbitol.
(c) Growth response to choline chloride.

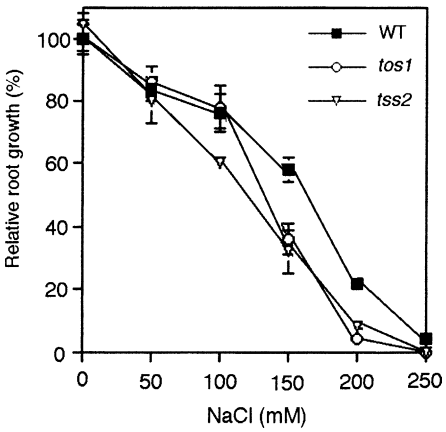

Figure 3. *tos1* seedlings are hypersensitive only to high concentrations of NaCl.
Root elongation of wild-type (WT) *tos1* and *tss2* seedlings was measured to quantify their sensitivities to NaCl inhibition as described in Figure 2. Root growth of NaCl-treated seedlings was expressed as a percentage relative to controls incubated on MS medium. Error bars represent SD ($n = 10$). The experiment was repeated at least thrice with similar results. The measurements from one representative experiment are presented.

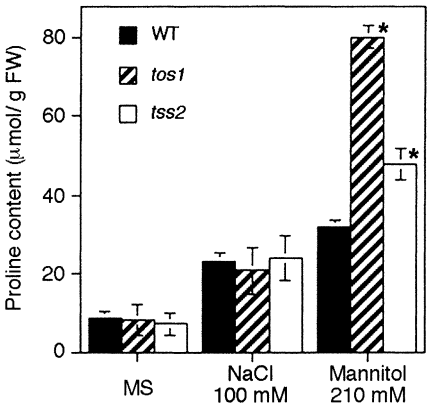

Figure 4. *tos1* and *tss2* seedlings over-accumulate proline after osmotic stress.
Seed of wild-type (WT) *tos1* and *tss2* were germinated and grown for 3 days on mannitol-free medium. Contents of proline were determined after incubating the seedlings for 3 days on control medium or medium supplemented with 100 mM NaCl or medium supplemented with 210 mannitol. The symbol (*) indicates that mean values are significantly different between wild type and mutants ($P < 0.05$). Error bars represent SD ($n = 10$). The experiment was repeated twice with similar results. FW, fresh weight.

Root growth and seed germination of tos1 *show reduced ABA sensitivity*

Osmotic stress elicited by water deficit or conditions of high salt cause an increase of the phytohormone ABA, whose perception and signalling is essential for stress tolerance (Leung and Giraudat, 1998). We determined whether the root growth of *tos1* was affected in its response to exogenous ABA. As shown in Figure 5(a), root growth of

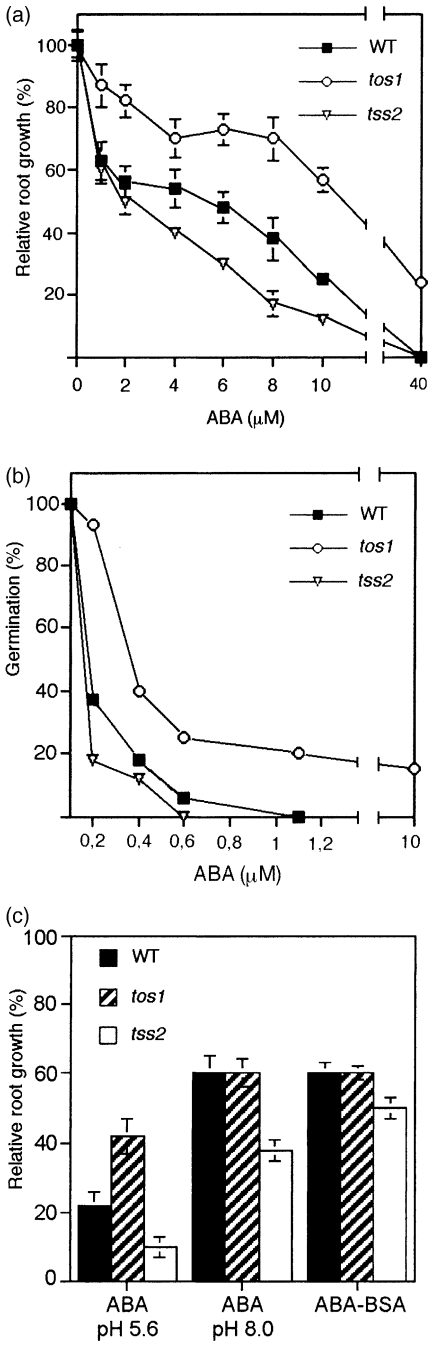

Figure 5. ABA response for wild-type *tos1* and *tss2*.
(a) ABA dose–response for root growth inhibition. Root elongation of wild-type (WT) *tos1* and *tss2* seedlings was measured to quantify their sensitivities to ABA inhibition as described in Figure 2. Root growth of ABA-treated seedlings was expressed as a percentage relative to controls incubated on MS medium. Error bars represent SD ($n = 10$). The experiment was repeated at least thrice with similar results. The measurements from one representative experiment are presented.
(b) ABA dose–response for germination inhibition. Seed were plated on medium supplemented with the indicated concentrations of ABA and incubated for 3 days at 26°C in the dark. The number of germinated seed was expressed as the percentage of the total number of seed plated (approximately 150). The experiment was repeated, at least, thrice with similar results. The result from one representative experiment is presented.

tos1 was less sensitive to ABA inhibition than that of wild type, in contrast to *tss2*, whose root growth was hypersensitive to ABA as described previously (Borsani *et al.*, 2001a). We determined whether the osmotic hypersensitivity exhibited by *tos1* seedlings co-segregated with ABA insensitivity. All 29 seedlings selected from the M2 population that did not exhibit hypersensitivity to mannitol showed wild-type growth in ABA-supplemented medium indicating that the osmotic hypersensitivity of *tos1* co-segregates with the ABA insensitivity. Because some of the seedlings that showed hypersensitivity to mannitol were severely damaged and unable to be recovered, we could not include them in the co-segregation study.

Endogenous ABA plays a major role in promoting seed dormancy in a number of plants (Leung and Giraudat, 1998). We investigated whether in addition to affecting ABA sensitivity in root growth, the *tos1* and *tss2* mutations also affect ABA response during germination. As shown in Figure 5(b), similar results to those obtained for root growth were obtained for seed germination. *tos1* seeds were less sensitive to ABA than wild-type seeds, whereas *tss2* seeds were hypersensitive to ABA. Despite their similar response to mannitol, we can now distinguish *tos1* and *tss2* by their opposite responses to ABA.

tos1 *has impaired intracellular ABA sensitivity*

Many lines of evidence indicate that there are multiple ABA perception and signalling mechanisms (Leung and Giraudat, 1998). Studies of the response to impermeant ABA–bovine serum albumin (ABA–BSA) conjugates (Jeannette *et al.*, 1999), and microinjection studies showed that ABA has both intra- and extracellular sites of action in plants (Leung and Giraudat, 1998). ABA is a weak acid (pK_a 4.7) that readily enters the cell when the extracellular pH is low. It has been determined that at pH 8, only 2% of the supplied ABA is found inside the cells (Jeannette *et al.*, 1999). As shown in Figure 5(c), no difference in ABA sensitivity between the wild type and the *tos1* mutant was found at pH 8 when the medium was supplemented with 20 μM ABA. However, *tss2* remained hypersensitive to ABA. To obtain direct evidence concerning the ABA perception locus affected in *tos1*, we used an ABA–BSA conjugate. ABA–BSA conjugate has been found to be stable and found not to

Figure 5. continued
(c) Seedlings were grown on a MS medium for 2 days. Later, the seedlings were transferred to a MS medium pH 5.6 (which is the pH value used throughout this study) containing 20 μM ABA (ABA pH 5.6) or to an MS medium pH 8.0 containing 20 μM ABA (ABA pH 8.0) and their growth was measured 2 days later. Root growth of ABA-treated seedlings was expressed as a percentage relative to controls incubated on MS medium. Seedlings were grown on MS medium for 2 days. Later, the seedlings were transferred to MS medium containing ABA–BSA conjugate 20 μM equivalent. Root growth of ABA–BSA-treated seedlings was expressed as a percentage relative to controls incubated on MS medium containing the same amount of BSA.

pass through the plasma membrane (Jeannette *et al.*, 1999). When we used ABA–BSA conjugate equivalent to 20 μM ABA, we obtained a reduction in growth for the wild type similar to the reduction previously obtained using pH 8, suggesting that the impermeant ABA–BSA conjugate was active (Figure 5c). The results using ABA–BSA conjugate were similar to those obtained previously using pH 8, and shows that the insensitivity of *tos1* to ABA disappears when the ABA is kept extracellular (Figure 5c). Hence, *tos1* sensitivity is altered in the intracellular ABA perception or signalling. Our data suggest that *tss2* is involved in extracellular ABA signalling while the intracellular role of *tss2* in ABA signalling cannot be ruled out.

The decreased ABA sensitivity of tos1 *is a basic cellular trait and is not caused by a defect in ABA synthesis*

To determine whether the reduced ABA sensitivity of *tos1* is a basic cellular trait or it is associated with specific cell and tissue types of intact plants, calli were generated from cotyledons of wild-type *tos1* and *tss2* mutants. The calli were then transferred to medium with and without ABA and their size monitored for several days. Figure 6(a) shows that in the absence of ABA, the growth of *tos1* and *tss2* calli was not different to that of wild type. In contrast, when grown on media containing 8 μM ABA, *tss2* and wild-type calli showed no noticeable growth while the growth of *tos1* callus was evident (Figure 6b). These results suggest that ABA insensitivity of *tos1* is a basic cellular trait expressed in cultured cells of the mutant.

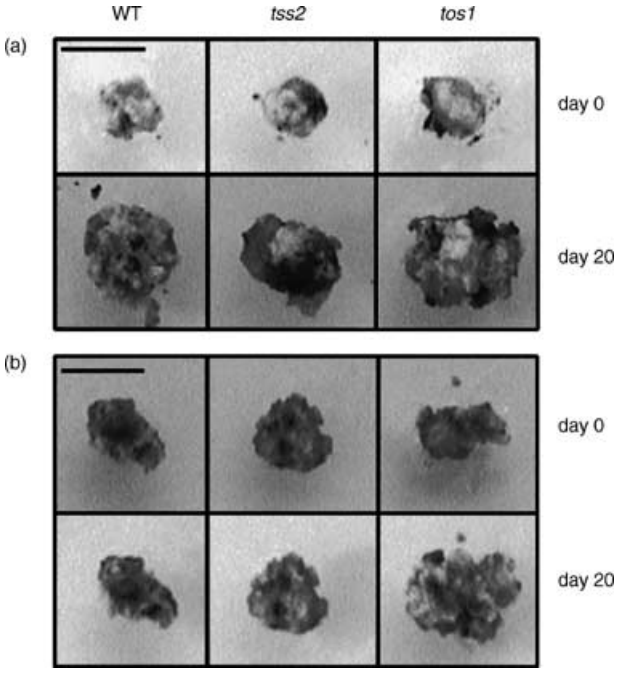

Figure 6. Callus tissue derived from *tos1* is less sensitive to ABA than callus tissue derived from wild type or *tss2*.
(a) The calli were photographed immediately after transfer to growth medium (day 0) and 20 days later (day 20). Bar = 5 mm.
(b) The calli were photographed immediately after transfer to growth medium supplemented with 8 μM ABA (day 0) and 20 days later (day 20). Bar = 5 mm.

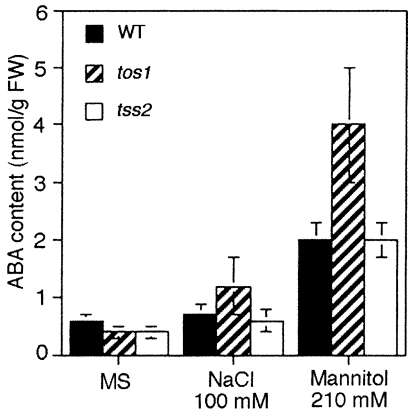

Figure 7. ABA accumulation in *tos1* seedlings.
Endogenous ABA concentrations in wild-type (WT) *tos1* and *tss2* seedlings. Seeds of wild-type *tos1* and *tss2* were germinated and grown for 3 days on MS medium. Resulting seedlings transferred to MS medium (MS), or MS medium supplemented with 100 mM NaCl and 210 mM mannitol. Endogenous ABA levels were determined after 3 days. The experiment was repeated at least thrice with similar results. The measurements from one representative experiment are presented.

The decreased sensitivity of *tos1* and the increased sensitivity of *tss2* to ABA could be caused either by abnormal ABA metabolism or by altered ABA signal transduction. Therefore, we determined the ABA concentrations in wild-type *tos1* and *tss2* seedlings before and after NaCl and mannitol stress. As shown in Figure 7, the endogenous ABA concentration in both *tos1* and *tss2* seedlings on a fresh weight basis were similar in control medium to the wild type. Upon NaCl stress, there was an increase in the endogenous ABA content in wild-type *tos1* and *tss2* seedlings but there were no statistically significant differences among them (Figure 7). Mannitol stress resulted in approximately four-fold increase in the ABA content for wild type and *tss2* seedlings. The ABA accumulation was higher in *tos1* seedlings after mannitol stress, with approximately eight-fold induction. We, therefore conclude that *tos1* is not an ABA-deficient mutant.

Discussion

Isolating the osmotic and ionic components of NaCl stress

A general consequence of salinity in glycophytes is the reduction of plant growth. A high NaCl concentration produces osmotic stress by decreasing the chemical activity of

water in the root medium. This reduces the uptake of water by the plant, resulting in a loss of cell turgor and a decrease in plant growth. This process occurs simultaneously with the absorption of Na^+ by the root which, being toxic for plants, may also decrease plant growth. Both the ionic and osmotic stresses act together and their damaging effects cannot be separated in wild-type plants. It has been generally accepted that osmotic is the main stress at low NaCl concentrations and that high NaCl concentrations are necessary for ionic toxicity (Munns, 1993; Newmann, 1997). A search for NaCl hypersensitive mutants in *Arabidopsis* and tomato only identified ionic mutants and established that K^+ nutrition is critical for Na^+ tolerance (Borsani *et al.*, 2001a; Zhu *et al.*, 1998). This suggests that at NaCl levels stressful for glycophytes such as *Arabidopsis* and tomato, the accompanying osmotic stress is still not a significant limiting factor. This is supported first by our studies showing that tomato root growth is more sensitive to mannitol than to iso-osmotic concentrations of NaCl, and secondly by the identification of *tos1*, a mutant that is hypersensitive to general osmotic stress but not to iso-osmotic concentrations of NaCl. As the decrease of water activity in the root medium is linearly related to NaCl concentration and the relation starts from 0 mM NaCl, absorption and loading of Na^+ and Cl^- into the vacuole may act as an osmotic agent that counteracts the decreased water activity till 150 mM NaCl (Niu *et al.*, 1995). Therefore, the use of NaCl to induce osmotic stress in plants can be complicated by the fact that only high NaCl concentrations seem to induce osmotic stress that is physiologically significant. It also supports the use of other osmotic stress agents rather than NaCl in the identification of plant osmotic mutants.

Is proline required for osmotolerance and salt tolerance?

Although an adaptive role for proline in mediating osmotic adjustment and protecting subcellular structures has been proposed, the role of proline in osmotolerance remains controversial. Many plants accumulate proline in response to environmental stresses that cause cellular dehydration and several reports have indicated a positive correlation between the accumulation of proline and osmotolerance in plants (Yoshiba *et al.*, 1997). Moreover, constitutive production of proline conferred osmotolerance in transgenic tobacco (Kishor *et al.*, 1995), and *Arabidopsis* transgenic lines that accumulated proline at significantly lower level than wild-type plants were hypersensitive to osmotic stress (Nanjo *et al.*, 1999). However, the lack of correlation between proline level and salt or osmotic tolerance in many other plant species has also led to the conclusion that proline accumulation is merely a by-product of the stress (Delaney and Verma, 1993; Hare and Cress, 1997; Moftah and Michel, 1987).

Genetic analysis of salt tolerance does not support the hypothesis that proline accumulation is a critical factor affecting salt tolerance. The *Arabidopsis sos1* mutant is 20 times more sensitive to NaCl than the wild type (Wu *et al.*, 1996), but the proline levels were nearly twice that of wild type after NaCl stress (Liu and Zhu, 1997). The salt-tolerant *Arabidopsis* mutants *rss* and *pst1* accumulated less proline than the wild type under NaCl stress (Tsugane *et al.*, 1999; Werner and Finkelstein, 1995). Therefore, proline accumulation could be consequence of the stress that correlates with the stress damage. The fact that *tos1* and *tss2* mutants over-accumulate proline after mannitol stress seems to support this notion, suggesting that proline content is not a factor limiting osmotic tolerance in tomato, at least in the short-term response.

TOS1*, a genetic locus essential for osmotic tolerance and ABA sensitivity*

The *tos1* mutant is hypersensitive to osmotic stress and exhibits reduced sensitivity to ABA. Phenotypic analysis of the *tos1* mutant indicated that the ABA insensitivity of *tos1* is a basic intracellular trait expressed at all stages analysed such as root growth, seed germination, and undifferentiated cells. However, the finding that *tos1* seedlings over-accumulate ABA under osmotic stress indicates that *tos1* does not fall into the group of mutants impaired in ABA biosynthesis (Taylor *et al.*, 2000). This over-accumulation can be explained by a failure in the feedback control of ABA homeostasis caused by faulty ABA signalling. The existence of negative feedback control of ABA is supported by the finding that transgenic tobacco ectopically expressing an ABA specific antibody and also that several *Arabidopsis* abi mutants exhibit increased ABA concentration (Artsaenko *et al.*, 1995; Koornneef *et al.*, 1984). Thus, the osmotic hypersensitivity of *tos1* is not due to reduced levels of ABA but more likely to an inadequate ABA-dependent signalling pathway necessary for osmotic tolerance (Shinozaki and Yamaguchi-Shinozaki, 1996).

Mutational analyses led to the identification of several loci that control ABA responsiveness (Leung and Giraudat, 1998). The *abi3*, *abi4* and *abi5* mutants show altered responses to ABA in germination but not during vegetative growth. Therefore, the proteins encoded by *ABI3*, *ABI4* and *ABI5* are thought to be mainly involved in seed development (Finkelstein and Lynch, 2000; Finkelstein and Somerville, 1990; Finkelstein, 1994; Koornneef *et al.*, 1984). In contrast, the *abi1* and *abi2* mutations affect ABA sensitivity in both seeds and vegetative tissues. Therefore, of the mutants found to have diminished sensitivity to ABA, only the *Arabidopsis abi1-1* and *abi2-1* dominant mutants are clearly affected in their ABA responses in vegetative tissues as the *tos1* recessive mutant reported here. However, intragenic revertants of the dominant mutant *abi1-1* were

hypersensitive to ABA, suggesting that ABI1, and most likely ABI2 proteins are negative regulators of ABA action (Gosti *et al.*, 1999). In contrast to *abi1* and *abi2*, the *tos1* mutation is recessive, indicating that the loss of *TOS1* function is responsible for the ABA insensitivity. Thus, the wild-type allele of *TOS1* is necessary for a proper ABA sensing and osmotic tolerance and is likely to be a positively acting component of the ABA signal transduction pathway. From the phenotype conferred by the *tos1* mutation, we can speculate on the function of the wild-type *TOS1* gene. A working hypothesis for ABA signalling is that ABA acts through a standard signal transduction pathway in which the binding of the hormone to a receptor elicits a transduction cascade (Bonetta and McCourt, 1998). The pleiotropic decrease in ABA sensitivity exhibited by *tos1* suggests that *TOS1* encodes an ABA receptor or an early signal transduction component. Because the *tos1* insensitivity is only apparent when intracellular ABA is elevated, the TOS1 protein must be involved in sensing or transmitting the ABA signal within the cell, and provides evidence of a specific intracellular ABA signalling pathway.

Use of mutant analysis shows that the network involving osmotic signalling can be rather complex with the multiplicity of intersecting signalling pathways (Foster and Chua, 1999; Ishitani *et al.*, 1997). However, advances in determining ABA perception and signalling have been obstructed by the paucity of mutants obtained (Møller and Chua, 1999). Our results using *tos1* and *tss2* indicate that novel ABA signal transduction components can be identified through their role in osmotic tolerance. Physiological analysis of these mutants have allowed the separation of the ionic and osmotic effects of NaCl as well as the determination of different mechanisms involved in osmotic tolerance.

Methods

Plant materials and growth conditions

M2 seed of the near-isogenic line of the tomato (*Lycopersicon esculentum*) cultivar Moneymaker homozygous for the *Cf-9* resistance gene after ethylmethane sulfonate mutagenesis were kindly provided by J.D.G. Jones (Sainsbury Laboratory, John Innes Centre, Norwich, UK). M2 seed families from individual M1 plants were kept separate to facilitate screening and to ensure that mutants isolated from different M2 seed lots were independent.

Seeds were surface sterilised with 40% (v/v) commercial bleach for 30 min and washed several times with sterile water. The seeds were first germinated until radicle emergence in sterile water because we found that this improved germination uniformity. The basal agar medium contained Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with 3% (w/v) sucrose and 0.7% (w/v) agar. The MS medium consists of the following: 1690 mg l^{-1} NH_4NO_3, 1900 mg l^{-1} KNO_3, 370 mg l^{-1} $MgSO_4{\cdot}7H_2O$, 170 mg l^{-1} $KHPO_4$, 378 $CaCl_2{\cdot}2H_2O$, 27.8 mg l^{-1} $FeSO_4{\cdot}7H_2O$, 37.2 mg l^{-1} disodium EDTA, 0.7495 mg l^{-1} NaI, 6.3 mg l^{-1} H_3BO_4, 16.9 mg l^{-1} $MnSO_4{\cdot}H_2O$, 8.6 mg l^{-1} $ZnSO_4{\cdot}7H_2O$, 0.25 mg l^{-1} $Na_2MO_4{\cdot}2H_2O$, 0.025 mg l^{-1} Cu $SO_4{\cdot}5H_2O$ and 0.025 mg l^{-1} $CoSO_4{\cdot}6H_2O$. The various agar plates used in this work were made by adding the appropriate amount of mannitol, sorbitol, choline chloride, NaCl and ABA to the molten basal medium. Light provided by cool-white fluorescent bulbs was at 50 μE m^{-2} sec^{-1} with 16 h of light at 22°C, 8 h of dark at 18°C and 70% relative humidity.

The osmotic potential generated by NaCl and mannitol was determined by the use of a cryoscopic osmometer (OSMOMAT 030, Gonotech, Berlin). The equivalence in osmotic potential between the molar concentrations of NaCl and mannitol in the media used in this study was calculated experimentally and can be estimated by the following expression:

$$[\text{Mannitol}] = 3.6 + 2.06\,[\text{NaCl}]$$

Isolation of tos1 *and genetic analysis*

Between 30 and 40 seeds from each M2 were screened for mannitol hypersensitivity mutants using the assay previously described (Borsani *et al.*, 2001a). Three-day-old seedlings with 2-cm-long roots were transferred from vertical agar plates onto a second agar medium that was supplemented with 150 mM mannitol. Seedlings from every individual family were arranged in rows. The root tips were marked, the plates were oriented vertically and root growths were registred 2 days later. Because we were analysing families obtained from a single M1 seed, we expected a segregation ratio 3 : 1 wild type to mutant for a recessive mutation and 1 : 3 wild type to mutant for a dominant mutation.

Growth measurements

For growth measurements, the same protocol described above for mutant isolation was used. Ten seeds were used per treatment, and three replicates were run for each treatment. Increases in root length were measured with a ruler after 2 days of treatment. The only modification was in the experiment described in Figure 1 in which the root growth was measured after 3 days.

Callus initiation and maintenance

Callus was induced from cotyledons as described previously (Sancho *et al.*, 1996). Routinely, calli were maintained on medium containing Murashige and Skoog's salts, Vitamin B_5 (Gamborg *et al.*, 1968), 4.6 μM α-naphtalene

acetic acid, 0.40 μM 2,4-dichlorophenoxyacetic acid and 0.5 μM 6-furfurylaminopurine (kinetin).

Determination of proline content

Proline was extracted and quantified as described previously (Borsani *et al.*, 1999).

Determination of ABA content

ABA was quantified by ELISA using the MAC 252 monoclonal antibody to free *cis, trans* (+) ABA (Quarrie *et al.*, 1988). The monoclonal antibody was supplied by S. Quarrie, John Innes Centre, Norwich, UK. ABA was extracted and quantified essentially as described previously (Walker-Simmons, 1987) with the following modifications. Seedlings were frozen immediately in liquid N_2 and powdered. The powdered samples (0.1 g) were suspended in 5 ml methanol containing 100 mg l^{-1} butylated hydroxytoluene and 0.05% (w/v) citric acid (0.05 g ml^{-1}). The extracts were stirred overnight and the supernatants dried in a Rotavapor. The dried residues were resuspended in 1 ml of 10% (v/v) methanol, centrifuged and the supernatants used for the immunoassay. Conjugated ABA–BSA was prepared according to Weiler (1979) and a solution of the conjugated ABA–BSA (7 mg ml^{-1}) was diluted 1/10 000. The monoclonal antibody used, MAC 252, was diluted 1/2000. The second rabbit antibody, anti-mouse Ig, conjugated with alkaline phosphatase was used at a 1/20 000 dilution. The product of the alkaline phosphatase reaction was measured in a spectrophotometer at 405 nm. Replicate ABA standards were assayed for each plate. As a control for ABA determination, we used the ABA-deficient *flacca* tomato mutant (Taylor *et al.*, 2000). The ABA content of this mutant was similar to the reported previously (data not shown).

Acknowledgements

The mutagenised M2 and *flacca* tomato seeds were generously supplied by Jonathan Jones and Aine Plant, respectively. We thank José Botella, Des Bradley, José Manuel Pardo, Iri Amaya and Josh Mylne for helpful discussions and critical reading of the manuscript. This work was supported by a grant from the Universidad de Málaga and Junta de Andalucía (grant AGR-168).

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